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# Retinoic acid is a potential negative regulator for differentiation of human periodontal ligament cells

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*Background and objectives:* Retinoic acid (RA) exerts a wide variety of effects on development, cellular differentiation and homeostasis in various tissues. However, little is known about the effects of RA on the differentiation of periodontal ligament cells. In this study, we investigated whether RA can affect the dexamethasone-induced differentiation of periodontal ligament cells.

*Methods and results:* Human periodontal ligament cells were differentiated via culturing in the presence of dexamethasone, ascorbic acid, and β-glycerophosphate for mineralized nodule formation, as characterized by von Kossa staining. Continuous treatment with all-*trans*-RA inhibited the mineralization in a dose-dependent manner, with complete inhibition over 1 μM RA. Other RA analogs, 9-*cis*-RA and 13-*cis*-RA, were also effective. Furthermore, addition of RA for just the first 4 days completely inhibited the mineralization; however, as RA was added at later stages of culture, the inhibitory effect was diminished, suggesting that RA had a phase-dependent inhibition of mineralization. RA receptor (RAR)-α agonist (AM-580), but not retinoid X receptor agonist (methoprene acid), inhibited the mineralization, and reverse transcription–polymerase chain reaction analysis revealed that RAR-α was expressed on the cells, suggesting that RAR-α was involved in the inhibitory mechanism. This inhibition was accompanied by inhibition of alkaline phosphatase activity; however, neither expression of platelet-derived growth factor (PDGF) receptor-α, PDGF receptor-β, or epidermal growth factor (EGF) receptor, nor phosphorylation of extracellular signal-regulated kinases triggered by PDGF-ascorbic acid or PDGF-BB was changed, as assessed by flow cytometry or western blot analyses.

*Conclusions:* These findings suggest that RA is a potential negative regulator for differentiation of human periodontal ligament cells.

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Retinoids are derivatives of retinal (vitamin A) that exert a wide variety of profound effects on vertebrate development, cellular differentiation and homeostasis in various tissues, both in embryos and in adults (1). The various biological effects of retinoic acid (RA) are mediated by two families of nuclear receptors, the RA receptor (RAR) family, comprising three isotypes, RAR-α, RAR-β, and RAR-γ, and the retinoid X receptor (RXR) family, also comprising three isotypes, RXR-α, RXR-β, and RXR-γ (2). The natural ligands for the RARs are the major physiological RA, all-*trans*-RA (ATRA), and its stereoisomers, 9-*cis*-RA and 13-*cis*-RA, whereas RXRs are activated by 9-*cis*-RA only (2). These receptors bind as RAR/RXR heterodimers or RXR/RXR homodimers to specific DNA sequences (retinoic acid response elements) in regulatory regions of target genes (2).

Among the effects of RA on various types of cells, RA has been shown to modulate osteoblast proliferation and differentiation, although it is suggested that its effects differ depending on the species, including mouse, rat, and human, or the differentiation stage of the cells being considered (3–15). The part played by RA in the development of dental tissue has been indicated by the results of studies on the administration of a vitamin A-deficient diet to rats, in which the periodontal ligaments were wider than those in the controls (16) and in which odontogenesis was disturbed through atrophy of odontoblasts and metaplasia of the enamel organ (17, 18). Furthermore, periodontal ligament cells of the rat contain cellular RA-binding protein, cytoplasmic protein that specifically binds RA (19). Retinaldehyde dehydrogenase-2, which is involved in the synthesis of RA, is distributed in blood vessels in the periodontal ligament of the rat (20).

Periodontal ligament cells are regarded as having the capacity to differentiate into cementoblasts or osteoblasts depending on need, and to form cementum or alveolar bone (21). Periodontal ligament cells *in vitro* have been shown to possess osteoblast-like properties, including a high level of alkaline phosphatase (ALP) expression, production of a cAMP in response to parathyroid hormone (22), and synthesis of bone-associated proteins in response to 1,25-dihydroxyvitamin $D_3$ (22). Furthermore, when cultured with ascorbic acid, dexamethasone, and β-glycerophosphate, periodontal ligament cells are capable of producing cementum-like mineralized nodules that are morphologically different from the bone-like mineralized nodules formed by osteoblastic cells (23, 24). However, little is known about the effects of RA on the differentiation of periodontal ligament cells.

The present study clearly showed that RA exhibited a phase-dependent inhibition of dexamethasone/ascorbic acid-induced mineral nodule formation by periodontal ligament cells, which was mediated via the RAR-α signaling pathway, and this inhibition was accompanied by inhibition of ALP activity.

## Material and methods

### Reagents

ATRA, 9-*cis*-RA, 13-*cis*-RA, ascorbic acid, β-glycerophosphate, dexamethasone, *p*-nitrophenyl phosphate, and Cell Dissociation Solution® were purchased from Sigma Chemical Co. (St. Louis, MO, USA). AM-580 and methoprene acid were from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA, USA). Phycoerythrin-conjugated monoclonal antibodies (mAb) for human platelet-derived growth factor (PDGF) receptor-α (αR1, mouse IgG2a), PDGF receptor-β (28D4, mouse IgG2a), epidermal growth factor (EGF) receptor (EGFR.1, mouse IgG2b) and isotype-matched control IgG conjugated with phycoerythrin were purchased from BD Biosciences PharMingen (San Diego, CA, USA). Human recombinant (r) PDGF-ascorbic acid and rPDGF-BB were obtained from R & D Systems Inc. (Minneapolis, MN, USA). Cell lysis buffer® was obtained from Cell Signaling Technology (Beverly, MA, USA). All other reagents were obtained from Sigma unless otherwise indicated.

### Cells

Human periodontal ligament cells were obtained, after receiving informed consent, from the periodontal ligaments of fully erupted third molar teeth of healthy individuals (aged between 16 and 23 years) having no clinical signs of inflammation in the periodontal tissues. Periodontal ligaments were dissected from the middle third of the root with a sharp blade, cut into small pieces, and cultured in tissue culture dishes containing a culture medium composed of α-Minimum Essential Medium (α-MEM) (Gibco BRL, Rockville, MD, USA) with 10% heat-inactivated fetal bovine serum (Flow Laboratories, McLean, VA, USA), 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B, with a medium change every 3 days until confluent cell monolayers formed. After confluency, the cells were passaged with 0.25% trypsin–0.1% EDTA. Periodontal ligament cells were used for the fourth and seventh passage in all experiments.

### Mineralized nodule formation

Confluent periodontal ligament cells in 24-well multiplates were cultured in α-MEM with 10% fetal bovine serum supplemented with ascorbic acid (50 μg/ml), dexamethasone (1 μM), and β-glycerophosphate (10 mM), with a medium change every 4 days in all experiments, and cultured up to the indicated days. For the treatment with RA analogs or RAR/RXR agonists, these were dissolved in ethanol, to a final percentage (v/v) of ethanol of 0.1% in all samples.

### Von Kossa staining

Periodontal ligament cells on 24-well multiplates were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline for 10 min, stained with 5% (w/v) silver nitrate in distilled water for 1 h, treated with 5% (w/v) sodium thiosulfate for 2 min, and then washed with distilled water. The stained periodontal ligament cells were digitally photographed with an Olympus IX70 microscope equipped with a digital imaging device, a Penguin 600 CL (Pixera Corp., Los Gatos, CA, USA), using the phase contrast mode, plus, the image of each entire plate was scanned with an Epson Scan ES-2200 (SEIKO EPSON Corp., Nagano, Japan). The images were converted to binary using NIH Image software, and the nodule areas stained with silver nitrate were quantified.

### Reverse transcription–polymerase chain reaction (RT–PCR) assay

Total cellular RNA was extracted from periodontal ligament cells cultured in a six-well multiplate by Isogen® (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription of the RNA samples to cDNA was done using a TaKaRa RNA PCR™ Kit (AMV) Version 2.1 (TAKARA BIO, Shiga, Japan). To transcribe the total RNA into cDNA, 1 μg of RNA, 250 U/ml reverse transcriptase XL isolated from avian

*Table 1.* Primer pairs used for polymerase chain reaction amplifications

| Gene (fragment) | Primer sequences | Denaturation/annealing/extension °C (s) | Cycle | Reference |
|---|---|---|---|---|
| RAR-α (438 bp) | 5′-CCCACCTCCGGCGTCAGCGTG-3′<br>5′-CACTGGCTTGACCATCGCAGACC-3′ | 94 (60)/61 (60)/72 (60) | 30 | 34 |
| RAR-β (435 bp) | 5′-GAGAGGTGGCATTGATCCAGG-3′<br>5′-GGCCTGGGCCAGCCTGACCTC-3′ | 94 (60)/63 (60)/72 (60) | 30 | 34 |
| RAR-γ (351 bp) | 5′-TTCGAGATGCTGAGCCCTAGCTTCC-3′<br>5′-CATGCCCACTTCGAAGCACTTCTGT-3′ | 95 (60)/58 (60)/72 (60) | 30 | 33 |
| RXR-α (327 bp) | 5′-GGCGCAGATGTGCTTGGTG-3′<br>5′-ATGCCACCCCCGCCACTGGGC-3′ | 94 (60)/63 (60)/72 (60) | 30 | 34 |
| RXR-β (111 bp) | 5′-GAAGCTCAGGCAAACACTAC −3′<br>5′-TGCAGTCTTTGTTGTCCC-3′ | 95 (60)/54 (60)/72 (60) | 30 | 33 |
| RXR-γ (361 bp) | 5′-CTCAGGAAAGCACTACGGGG-3′<br>5′-CAGGGTCATTTGTCGAGTTC-3′ | 95 (60)/54 (60)/72 (60) | 30 | 33 |
| β-Actin (336 bp) | 5′-ATTGGCAATGAGCGGTTCCGC-3′<br>5′-CTCCTGCTTGCTGATCCACATC-3′ | 94 (60)/55 (60)/72 (60) | 30 | |

RAR, retinoic acid receptor; RXR, retinoid X receptor.

myeloblastosis virus, 5 mM $MgCl_2$, 1 mM dNTP mixture, 1000 U/ml RNase inhibitor, and 2.5 μM Random 9 mer were mixed in a PCR buffer (total volume of 20 μl). The reaction mixture was incubated for 10 min at 30°C, then 30 min at 42°C, followed by 5 min at 95°C. The primers used for PCR are given in Table 1. The PCR mixture contained 5 μl of the cDNA mixture, 2 μl of 10 × PCR buffer, 0.2 mM deoxynucleoside triphosphate, 50 pmol of each primer, and 0.1 μl of Ex Taq DNA polymerase (Takara, Tokyo, Japan) in a total volume of 20 μl. Amplification was performed in an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the cycle program shown in Table 1. Amplified samples were visualized on 2.0% agarose gels stained with ethidium bromide, and photographed under UV light.

### Alkaline phosphatase assay

Periodontal ligament cells cultured on 24-well multiplates were washed with phosphate-buffered saline. The activity was assayed by adding 1 mg/ml of *p*-nitrophenyl phosphate as a substrate in 0.1 M glycine buffer (pH 10.4) containing 1 mM $MgCl_2$ in a final volume of 1 ml for the indicated time at 37°C. Supernatants were harvested, mixed with NaOH (final 0.25 N) to stop the reactions, and read spectrophotometrically at 405 nm.

### Cell proliferation assay

The number of periodontal ligament cells was determined using a cell counting Kit-8 (Dejindo Laboratories, Kumamoto, Japan) composed of 5 mM WST-8 (2-(2-methoxy-4 nitrophenyl-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), 0.2 mM 1-methoxy PMS, and 0.15 M NaCl. Periodontal ligament cells cultured on 24-multiplates were washed with PBS followed by the addition of 1 ml of 10% of WST-8 solution. After incubation for 1 h at 37°C, the reaction was stopped by the addition of 100 μl of 0.1 M HCl into the well. The supernatants were measured at 450nM using a VERSAmax™ Tunable Microplate Reader (Molecular Devices Co., Sunnyvale, CA).

### Flow cytometry analysis

Periodontal ligament cells in 24-well multiplates were collected using Cell Dissociation Solution® (no-enzymatic) to avoid possible proteolysis destruction of cell surface proteins, and processed by being passed through a nylon mesh filter (94-μm mesh size). A total of $1 \times 10^5$ cells were stained with phycoerythrin-conjugated mAb or an isotype-matched control IgG at 4°C for 20 min. Staining was analyzed on a FACScan® (BD Biosciences, San Jose, CA, USA). The arithmetic mean was used in the computation of the mean fluorescence intensity.

### Western blotting

Confluent monolayer cells cultured in PRIMARIA™ EASYGRIP™ 35-mm tissue culture dishes (BD Bioscience Discovery Labware, Bedford, MA, USA) were starved with fetal bovine serum for 24 h, and then stimulated with 50 ng/ml PDGF in 1 ml of α-MEM for 15 min at 37°C. Cells were harvested with 100 μl of cell lysis buffer® using a cell scraper and incubated on ice for 30 min, followed by centrifugation at 12,000 *g* at 4°C for 10 min. The supernatants (25 μl) were solubilized with Laemmli sample buffer at 100°C for 5 min, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10%), and transferred to a polyvinylidene difluoride membrane (ATTO Co., Tokyo, Japan) using a semidry transblot system (ATTO). The blot was blocked with 0.5% (w/v) non-fat dried milk and 0.1% (v/v) Tween 20 in phosphate-buffered saline at 4°C overnight or at room temperature for 1 h, followed by incubation for 1 h at room temperature with rabbit anti-phospho extracellular signal-regulated kinases (ERK) polyclonal Abs (Cell Signaling Technology) at 1:1000. The blot was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology) at 1:2000 for 1 h at room temperature. The blot was then treated with western blotting detection reagent ECL Plus® (Amersham Pharmacia Biotech Inc.,

Piscataway, NJ, USA) to produce a chemiluminescence, as instructed by the manufacturer. The detected blot was exposed to Polaroid™ film using an ECL mini-camera. Phospho-ERK antibodies on membranes were removed with a Re-Blot Plus Western Blot Recycling Kit® (Chemicon International, Inc., Temecula, CA, USA) according to the manufacturer's instructions, and the membrane was reprobed with corresponding rabbit anti-ERK polyclonal Abs (Cell Signaling Technology) at 1:1000. The molecular weight of the proteins was estimated by comparison with the position of the standard (Bio-Rad Laboratories).

### Statistical analysis

All experiments in this study were performed at least three times to test the reproducibility of the results, and the representative findings are shown. In some experiments, experimental values are given as means ± SE. The significance of differences between two means was evaluated by one-way ANOVA. *p*-values less than 0.05 were considered significant.

## Results

### Retinoic acid inhibits dexamethasone/ascorbic acid-induced mineral nodule formation by periodontal ligament cells

The culture of periodontal ligament cells with dexamethasone/ascorbic acid for 20 days induced mineral nodule formations, as assessed by von Kossa staining (Fig. 1A). The continuous treatment with ATRA inhibited the formation in a dose-dependent manner, and complete inhibition was observed with greater than 1 μM ATRA (Figs 1A and B). Next, we investigated the possibility that there may be a sensitive phase in ATRA treatment for inhibiting the mineralization during the culture. RA was administered for 4 or 8 days to the culture at the indicated time (Fig. 2A) after the initiation of dexamethasone/ascorbic acid stimulation. In this experiment, mineral nodules were first detected at day 18, and the number and size gradually increased. Figures 2(B) and (C) show that the treatment with ATRA for just the first 4 days (sample no. 5) inhibited the formation down to the level of the background. As ATRA was added at later stages of culture, the inhibitory effect was diminished (sample nos 6–8), suggesting that ATRA has a phase-dependent inhibition of mineral nodule formation. Next, we examined whether retinoids other than ATRA have the ability to inhibit mineral nodule formation. As ATRA-treatment for the first 4 days was found to be sufficient for inhibiting the mineralization, cells were pretreated with RA for 4 days and

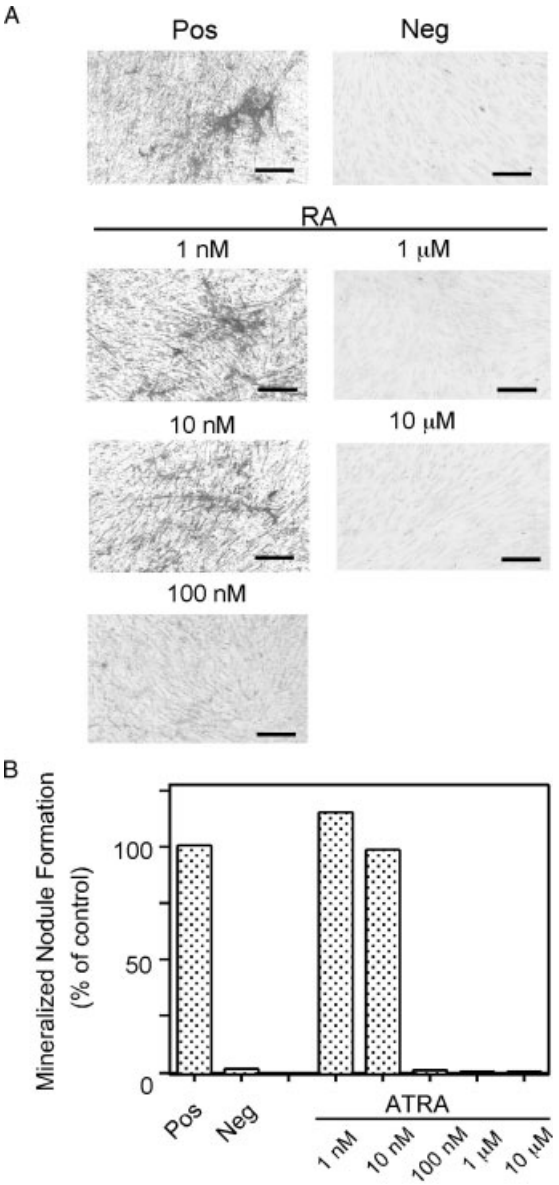



*Fig. 1.* Effect of all-*trans*-retinoic acid (ATRA) on mineralized nodule formation by periodontal ligament cells. (A) Confluent periodontal ligament cells were cultured in medium containing dexamethasone, ascorbic acid, and β-glycerophosphate for 21 days in the absence (Pos) or presence of the indicated concentration of retinoic acid (RA), and subjected to von Kossa staining. For the negative control (Neg), periodontal ligament cells were cultured in medium containing β-glycerophosphate only. (B) The images of (A) were converted to binary using NIH Image software, and the nodule areas stained with silver nitrate were quantified. Findings are representative of three independent experiments with three different donors. Scale bars represent 500 μm.

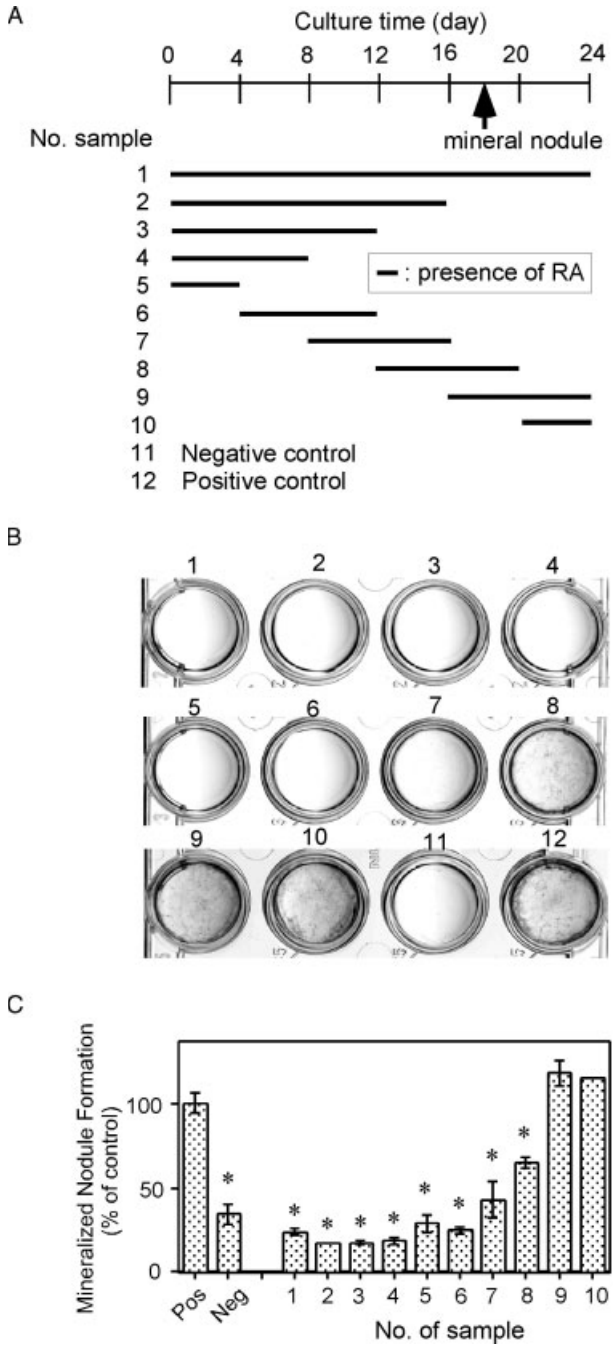



*Fig. 2.* Effect of all-*trans*-retinoic acid (ATRA) at different stages of culture on mineralized nodule formation by periodontal ligament cells. Confluent periodontal ligament cells were cultured in medium containing dexamethasone, ascorbic acid, and β-glycerophosphate for 24 days. (A) Retinoic acid (RA) (1 μM) was administered to the culture for the indicated periods after the initiation of dexamethasone/ascorbic acid stimulation, and (B) subjected to von Kossa staining. The negative control (Neg) was cultured in medium containing β-glycerophosphate only. (C) The images of (B) were converted to binary using NIH Image software, and the nodule areas stained with silver nitrate were quantified. Representative findings of three independent experiments are shown as the mean ± SE of triplicate assays. Significance is shown (*$p < 0.05$ vs. positive control).

then stimulated with dexamethasone/ascorbic acid. As shown in Fig. 3, ATRA and its stereoisomers 9-*cis*-RA and 13-*cis*-RA inhibited the formation in a dose-dependent manner, with substantially equal potency.

### Retinoic acid receptor-α is involved in the inhibition of mineral nodule formation by periodontal ligament cells

To clarify the mechanism of the inhibition of mineralization by RA, selective agonists were used instead of ATRA. AM-580 is a selective agonist for RAR-α with no affinity for RXRs, and methoprene acid is a selective agonist for RXRs with no binding to RARs, unlike 9-*cis*-RA. As shown in Fig. 4, RAR-α agonist, but not RXRs agonist, inhibited the formation with a slightly stronger potency than ATRA, although not significantly. We verified the expression of RARs and RXRs on periodontal ligament cells by RT–PCR. Total RNA was purified from the periodontal ligament cells before and after stimulation with dexamethasone/ascorbic acid for 12 days, which were referred to as day 0 and day 12, respectively. Figure 5 shows that both day 0 and day 12 periodontal ligament cells expressed RAR-α, -β, -γ, and RXR-β, but not RXR-α or -γ. These findings suggest that RAR-α is involved in the inhibition of mineral nodule formation by periodontal ligament cells and, furthermore, that the phase-dependent inhibition by RA might not be due to dependence on RAR and RXR expressions.

### Inhibition of mineral nodule formation by all-*trans*-retinoic acid is accompanied by inhibition of alkaline phosphatase activity on periodontal ligament cells

We examined whether ALP activity may be regulated by ATRA treatment, as mineralization is known to be linked closely to ALP activity. Figure 6(A) shows that the differentiation of periodontal ligament cells in our experiment was characterized by two parameters, mineral nodule formation and ALP activity. The culture period was tentatively divided into four stages: initiation of induction of ALP activity (stage I, days 0–5), progressive induction of ALP activity (stage II, days 5–10), maximal ALP activity and initiation of mineralization (stage III, days 10–15), and progressive mineralization

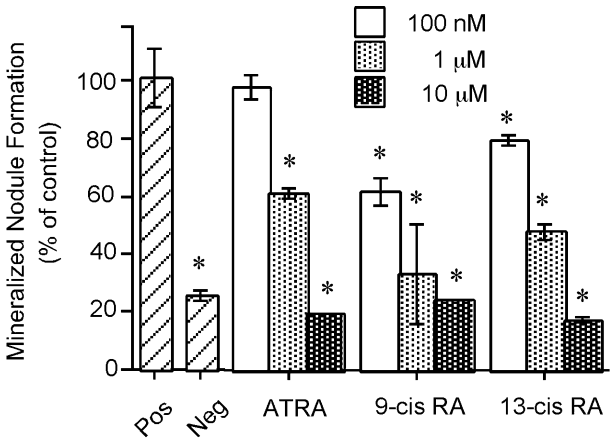



*Fig. 3.* Effect of all-*trans*-retinoic acid (ATRA) and its isomers on mineralized nodule formation by periodontal ligament cells. Confluent periodontal ligament cells were pretreated with the indicated concentrations of retnoic acid (RA) analogs for 4 days without dexamethasone/ascorbic acid, followed by stimulation with dexamethasone/ascorbic acid and β-glycerophosphate for 21 days. Cells were subjected to von Kossa staining, and nodule areas stained were quantified using NIH Image software. The negative control (Neg) was cultured in medium containing β-glycerophosphate only. Representative findings of three independent experiments are shown as the mean ± SE of triplicate assays. Significance is shown (*$p < 0.05$ vs. positive control).

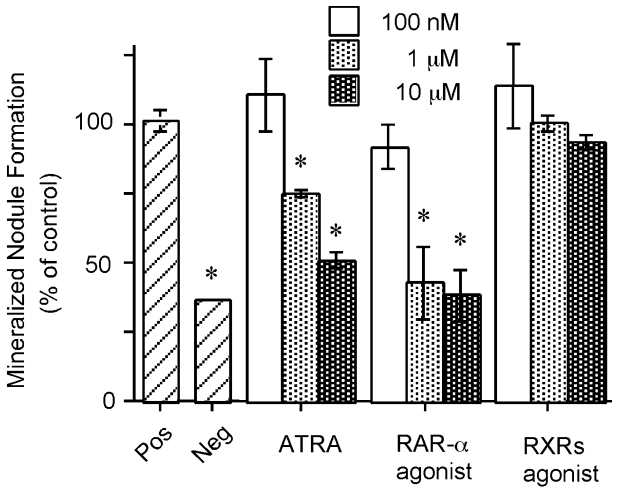



*Fig. 4.* Effect of retinoic acid receptor (RAR)/retinoid X receptor (RXR) agonists on mineralized nodule formation by periodontal ligament cells. Confluent periodontal ligament cells were pretreated with the indicated concentrations of all-*trans*-retinoic acid (ATRA) or RAR/RXR agonists for 4 days without dexamethasone/ascorbic acid, followed by stimulation with dexamethasone/ascorbic acid and β-glycerophosphate for 21 days. Cells were subjected to von Kossa staining, and nodule areas stained were quantified using NIH Image software. The negative control (Neg) was cultured in medium containing β-glycerophosphate only. Representative findings of three independent experiments are shown as the mean ± SE of triplicate assays. Significance is shown (*$p < 0.05$ vs. positive control).

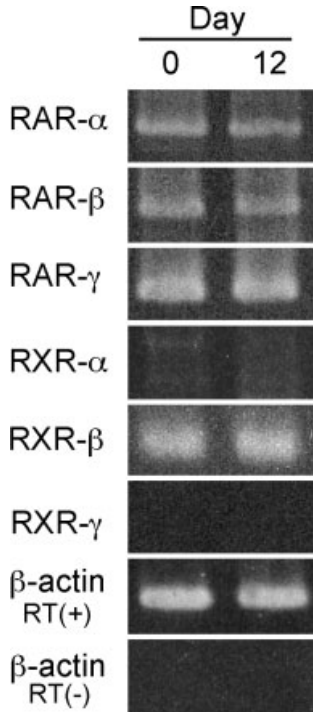
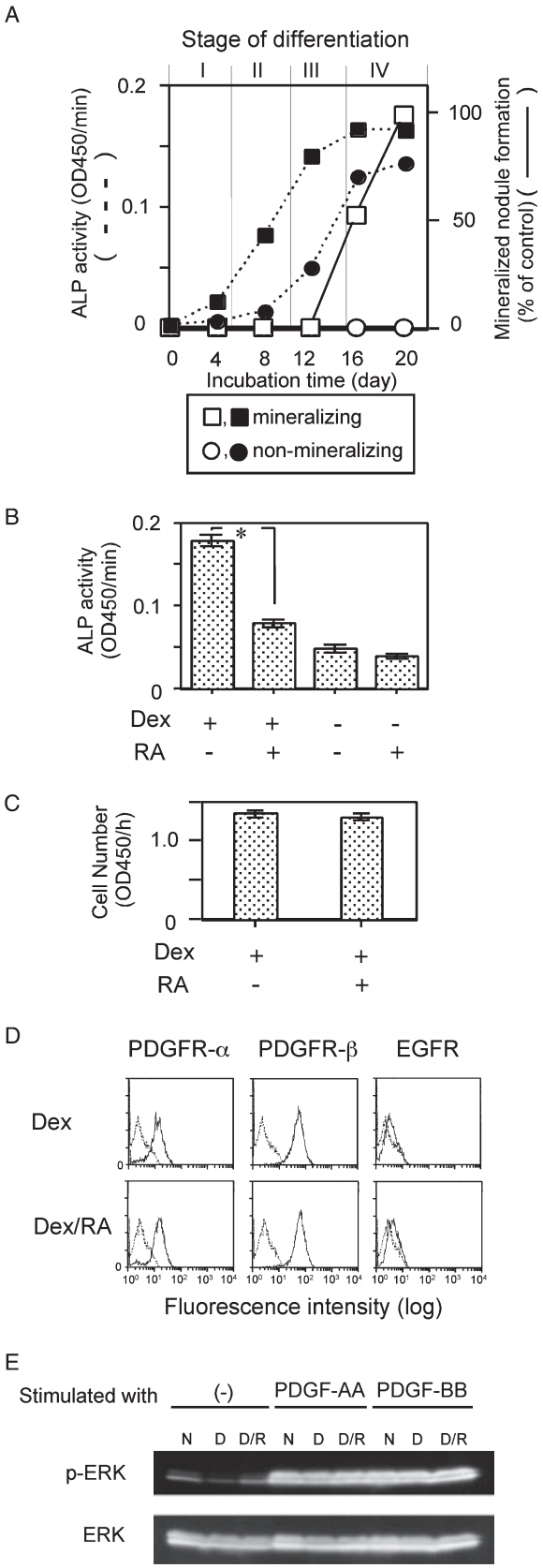



*Fig. 5.* Expression of retinoic acid receptor (RAR) and retinoid X receptor (RXR) mRNA on periodontal ligament cells. Confluent periodontal ligament cells (referred to as day 0) were cultured in medium containing dexamethasone, ascorbic acid, and β-glycerophosphate for 12 days (referred to as day 12). Total cellular RNA was extracted from the culture at day 0 and day 12. Expressions of RAR-α, -β, and -γ, and RXR-α, -β, and -γ mRNA were assessed by reverse transcription–polymerase chain reaction. Findings are representative of three independent experiments.

(stage IV, days 15–20). As high ALP activity and initiation of mineralization (stage III) would indicate an appropriate stage at which to examine the effects of ATRA on ALP activity, cells were stimulated with dexamethasone/ascorbic acid for 12 days, after pretreatment with ATRA for 4 days. As shown in Fig. 6(B), ALP activity on dexamethasone/ascorbic acid-stimulated periodontal ligament cells was significantly inhibited by ATRA treatment, and furthermore, ALP activity on unstimulated cells was also slightly inhibited, but not significantly. RA pretreatment of cells did not show any significant effects on cell numbers (Fig. 6C). Next, we examined whether growth factor receptors may be regulated by ATRA treatment. Among those receptors, PDGF receptor but not EGF receptor is involved in mineral nodule formation by PDL cells *in vitro* (25). Cells were treated with/without ATRA for 4 days, and then stimulated with dexamethasone/ascorbic acid for 12 days. As shown in Fig. 6(D), none of the receptor expressions were changed, namely, PDGF receptor-α and -β were expressed at the same level, and no EGF receptor was expressed in any




periodontal ligament cells. However, there is still the possibility that the signaling functions of the PDGF receptors were different. Therefore, we examined whether ERK is phosphorylated when triggered by PDGF-AA and PDGF-BB, which have a selective affinity for PDGF receptor-α and a similar affinity for both receptors (26), respectively, by using western blotting analysis. As shown in Fig. 6(E), PDGF-AA and PDGF-BB induced ERK1/2 phosphorylations in periodontal ligament cells regardless of

*Fig. 6.* Effect of retinoic acid (RA) on the expressions of alkaline phosphatase (ALP) activity and growth factor receptor on periodontal ligament cells. (A) Confluent periodontal ligament cells were cultured in a mineralizing or non-mineralizing condition for 20 days. At the time indicated, the cells were subjected to von Kossa staining, or ALP activity on periodontal ligament cells was measured as described in the Materials and Methods. (B, C, D and E) Confluent periodontal ligament cells were pretreated with 1 μM of RA for 4 days without dexamethasone/ascorbic acid, followed by stimulation with dexamethasone/ascorbic acid and β-glycerophosphate for 12 days. (B) ALP activity on periodontal ligament cells was measured as described in Materials and Methods. (C) Cell numbers were counted as described in materials and methods. (D) The cells were collected, stained with anti-platelet-derived growth factor receptor-α (PDGFR-α) and (PDGFR-β), and epidermal growth factor receptor (EGFR) mAb, and analyzed using flow cytometry. (E) The cells were starved with fetal bovine serum for 24 h in α-Minimum Essential Medium (α-MEM), and stimulated with 50 ng/ml of PDGF-AA or PDGF-BB for 15 min. Cell lysates were analyzed by western blotting with phospho-specific p44/42 ERK1/2 (Thr202/Tyr204) antibody to detect the phosphorylation of ERK1/2. Antibody against total p44/42 ERK1/2 was used as a control. The molecular weights (kDa) of ERK1 and ERK2 were 42-kDa and 44-kDa, respectively (N:non stimulation; D: dexamethasone; R: retinoic acid). Findings are representative of three independent experiments (A, D and E). Representative findings (B and C) of three independent experiments are shown as the mean ± SE of triplicate assays. Significance is shown (*$p < 0.05$ vs. respective control).

pretreatment with RA, indicating that RA affected neither the expressions nor the signaling functions of PDGF receptors on periodontal ligament cells.

## Discussion

In the present study, we clearly demonstrated that RA inhibited the expression of ALP and the mineralization of dexamethasone/ascorbic acid-stimulated periodontal ligament cells. ATRA has been reported to have differential effects on ALP activity in osteoblastic cells. ALP activity has been induced by ATRA in the culture of rat osteoblastic cell lines, RCT-1 (6) and UMR-201 (7–9), as well as in murine osteoblastic cell lines, MC3T3-E1 (10) and C3H-10T1/2 (11, 12). On the other hand, it has been inhibited by ATRA in the culture of murine osteoblastic cell line 3/A/1D-1M (3), rat osteosarcoma, ROS 17/2A (13), rat osteoblastic cell line UMR 106-06 (14), fetal rat calvarial cells (15), and human osteoblastic cell line SV-HFO (4, 5). This radically opposite effect is suggested to be due to the stage of differentiation (3, 6), based on the evidence that RA stimulates ALP activity in immature cells expressing a weak basal ALP activity (6–12), and inhibits the activity in phenotypically mature cells (3–5, 13–15). This hypothesis is further supported by our findings that ALP activity was inhibited by ATRA in the presence of dexamethasone, an inducer of osteoblastic differentiation and ALP expression *in vitro*. On the other hand, it has been reported that ATRA enhanced ALP activity in a culture system in the absence of Dex stimulation (27). In our experiment, however, there was no statistically significant change of ALP activity in the absence of Dex. It might be difficult to compare these results directly since the methods in their report differed from ours, such as the cell culture time (4 days vs. 12 days) and the ALP assay (using whole cell lysate vs. cell surface). Moreover, it is generally accepted that the population of periodontal ligament cells is heterogeneous, including undifferentiated mesenchymal cells and osteogenic progenitors committed to become osteblasts or cementoblasts (28). Accordingly, this differential effect of ATRA on ALP expression on periodontal ligament cells might be due to the stage of differentiation.

In this study, we showed that RAR-α could be involved in the inhibition of mineralization. ATRA, 9-*cis*-RA, and 13-*cis*-RA have been reported to be able to bind to RAR-α, -β, and -γ with variable affinity (29); however, 9-*cis*-RA is the most effective retinoid for RAR-α activation, followed by 13-*cis*-RA and ATRA (29). For RAR-β, ATRA is the most effective ligand, 13-*cis*-RA is also a good transactivator, and 9-*cis*-RA is less effective (29). For RAR-γ, ATRA is the most effective retinoid, followed by 13-*cis*-RA and 9-*cis*-RA (29). Here, we showed that 9-*cis*-RA had a relatively stronger effect for the inhibition at a lower (100 nM) concentration, compared with that by ATRA or 13-*cis*-RA, suggesting that RAR-α may be more closely related to the inhibition. This would be consistent with the previous report that the inhibitory effect of ATRA on the mineralization of osteoblasts is mediated by the activation of RAR-α and/or RAR-β, but not of RAR-γ, using selective agonists for RAR-α, -β, and -γ (7).

We showed that, the mineralization of periodontal ligament cells was inhibited more effectively by the addition of ATRA at earlier stages of the culture, compared with that at later stages of the culture (Fig. 2). As the RAR isotypes exert different function regarding mineralization (7), one possible mechanism might be due to different levels of RAR expressions at different stages of culture. However, our findings showed that periodontal ligament cells from both the early stage of culture (day 0) and the late stage of culture (day 12) expressed mRNA of RAR-α, -β, and -γ, although it was hard to accurately quantify the RAR expressions with this assay (Fig. 5), suggesting that there might be another RA-indirect mechanism or the existence of another nuclear receptor for RA, such as peroxisome proliferator-activated receptor β/δ (30).

Signaling from PDGF receptor is required in mineral nodule formation by periodontal ligament cells (25). EGF receptor is suggested to be expressed on undifferentiated periodontal ligament cells, to act as a negative regulator of osteoblastic differentiation in periodontal ligament cells (31), and to be down-regulated on differentiation *in vitro* (31). However, our findings showed that ATRA affected neither the expressions nor the signaling functions of PDGF receptors, and did not induce EGF receptor on periodontal ligament cells (Figs 6D and E), suggesting that these receptors pathways are unlikely to be involved in the inhibition by RA.

The periodontal ligament is cellular connective tissue situated between the roots of the teeth and the alveolar bone. An important feature of the periodontal ligament is the markedly uniform preservation of periodontal width over time, the failure of which has been implicated in teeth ankylosis, despite exposure to osteogenic stimulation under various circumstances (28, 32). These findings suggest that RA is an important molecule in the mechanisms of maintaining periodontal homeostasis.

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